

CASE REPORT

Does Immunotherapy Protect Equines from Reinfection by the Oomycete *Pythium insidiosum*?[▽]

Carlos E. P. Santos,¹ Luiz C. Marques,² Régis A. Zanette,³
Francieli P. K. Jesus,⁴ and Janio M. Santurio^{3*}

Programa de Pós-graduação em Medicina Veterinária, Faculdade de Ciências Agrárias e Veterinárias (UNESP), Jaboticabal-SP, 14884-900, Brazil¹; Departamento de Clínica e Cirurgia Veterinária, Faculdade de Ciências Agrárias e Veterinárias (UNESP), Jaboticabal-SP, 14884-900, Brazil²; Programa de Pós-Graduação em Farmacologia, Universidade Federal de Santa Maria (UFSM), Santa Maria-RS, 97105-900, Brazil³; and Programa de Pós-Graduação em Ciências Veterinárias, Universidade Federal de Porto Alegre (UFRGS), Porto Alegre-RS, 91540-000, Brazil⁴

Received 6 May 2011/Returned for modification 17 May 2011/Accepted 15 June 2011

A cutaneous *Pythium insidiosum* reinfection was diagnosed in an equine in Brazil. Lesions with focal presentation appeared 2 years apart. The first infection and even immunotherapy were not likely to develop enough immune response to prevent reinfection. The use of adjuvants should be considered in the immunotherapy of pythiosis.

CASE REPORT

The case occurred in May 2007 in the Pantanal wetlands, Brazil. The animal showed a single fast-growing lesion in the left pelvic limb with approximately 45 days of evolution. The lesion in the shin region was moist, hemorrhagic, ulcerated, and slightly elliptical in shape, with irregular borders and a diameter of approximately 15 cm. Necrotic foci intermingled with sinuses containing yellowish firm structures measuring 2 to 5 mm, referred to as “kunkers,” were observed throughout the lesion (4, 13). Vital signs were within normal limits, although the animal was moderately emaciated.

One biopsy specimen was fixed in 10% neutral buffered formalin and stained by the hematoxylin and eosin (H&E) and Gomori methenamine silver (GMS) techniques. Irregularly ramified, scarcely septate hyphae with thick brown walls were visualized with GMS staining (Fig. 1A). Hypha-like structures surrounded by irregular, eosinophilic material could be observed in the necrotic areas and corresponded to Splendore-Hoeppli reactions (H&E) (Fig. 1B). A second biopsy specimen was refrigerated and used for PCR (1). Serum analyzed by enzyme-linked immunosorbent assay (ELISA) tested positive for pythiosis (14). The animal underwent five doses of a vortexed immunotherapy product (Pitium-vac; Mycological Research Laboratory [LAPEMI/UFSM]; Brazilian Agricultural Research Corporation [Embrapa-Pantanal]) at 14-day intervals, according to the manufacturer's instructions and information from previous studies (12, 14). Remission of signs and

weight gain were observed after 4 months of follow-up, when the animal was considered cured; i.e., it showed complete healing of the wound and negative ELISA values (Fig. 2A and B).

Two years later, in December 2009, a new lesion with a clinical course of 30 days was observed in the ventral abdomen of the animal. The ulcerated area measured around 20 cm in diameter, and the perilesional edema around 60 cm. Serosanguinolent discharge and a great quantity of “kunkers” of approximately 2 mm inside the sinuses were also observed. Diagnostic and follow-up procedures were the same as described earlier, and the animal was successfully cured following four doses of immunotherapy (Fig. 3A and B). Culture of *Pythium insidiosum* is time sensitive, which probably impaired the isolation of the agent in this study.

Pythiosis insidiosus, a disease caused by the oomycete *P. insidiosum*, often causes lesions in horses and other mammals, including humans (2, 13). Equines are the most affected, without having, to our knowledge, a predisposing epidemiological factor (4). Cutaneous/subcutaneous lesions affecting mainly the distal extremities of limbs and the ventral portion of the thoracoabdominal wall are usually observed, probably due to increased contact with water containing motile zoospores produced by the agent (2, 7).

The treatment of infections caused by *P. insidiosum* in animals and humans is varied and complicated because of some characteristics of the agent, such as the composition of the cell wall and the lack of ergosterol in the cytoplasmic membrane, ergosterol being the target of action for the majority of antifungal drugs available (4, 11). Radical surgery still is the treatment of choice and provides satisfactory results, although it

* Corresponding author. Mailing address: Laboratório de Pesquisas Micológicas, Department of Microbiology and Parasitology, Prédio 20, Sala 4139, Universidade Federal de Santa Maria, Brazil 97105-900. Phone and fax: 55 55 3220-8906. E-mail: janio.santurio@gmail.com.

[▽] Published ahead of print on 29 June 2011.

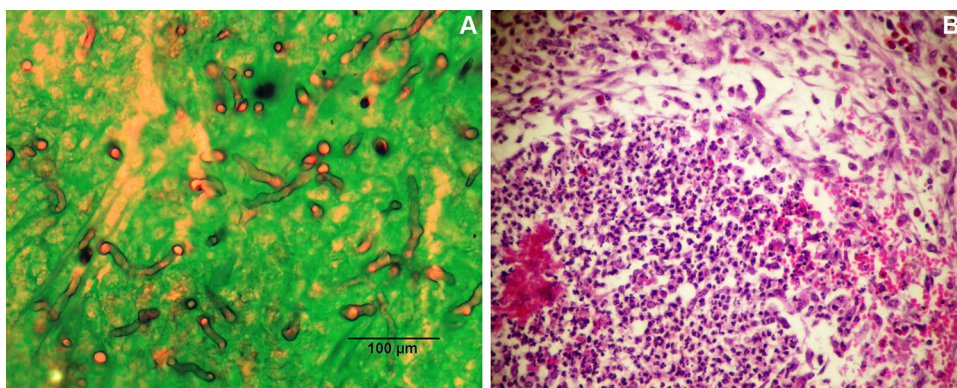


FIG. 1. (A) Typical silver-stained hyphae of *P. insidiosum*; (B) necrotic area surrounded by eosinophilic sleeves of Splendore-Hoeppli material showing hyphal ghosts (H&E, 20× objective).

can be complicated, especially when limbs are affected. Currently, immunotherapy has been applied, increasing the therapeutic success of surgical procedures or simply with clinical curative effects (8, 13). Actions such as debridement and burning, common empirical treatments in the Pantanal wetlands of Brazil, where equine pythiosis is considered endemic, are sometimes effective, although some farmers report recurrent episodes elsewhere (5). Most animals frequently cannot stand up, which usually leads to anemia and severe dehydration. If not successfully treated, the disease becomes chronic and can lead to death, although atypical cases with quiescent lesions and without emaciation have been reported in the Pantanal region (5).

Both the first and the second pythiosis infections occurred during the season most commonly reported by other authors, although the first occurred at the end and the second at the beginning of the summer (5, 13). According to Leal et al. (5), the lack of systematic studies makes it impossible to conclude whether horses which had been infected in previous years and were given cutting and burning treatments developed some degree of immunity. Indeed, the existence of an individual predisposition factor or difference in resistance among individuals is yet to be discovered. Such data are consistent considering the reinfection of the animal in our study, 2 years after the first presentation and immunotherapeutic treatment. The mechanisms by which immunotherapy against *P. insidiosum* works are based mainly on cellular response, a switch of initial eosinophilic to mononuclear response (4, 13). Antigens present in the immunogen are distinct from those triggered

during natural infection, and release mediators will activate cytotoxic lymphocytes and macrophages that eventually could eliminate *P. insidiosum* hyphae from the infected tissues (5, 12, 13).

The efficacy of the immunotherapeutic treatment was demonstrated in both events. Notably, the first presentation (appendicular injury) had a slower response, whereas the second presentation (abdominal injury) was cured with fewer applications, in spite of the greater severity and size of the lesion. Two hypotheses could explain the differences observed in the immune response. First, although the injury in the second episode was apparently more severe because of the size and state of emaciation, it was more recent. It is of utmost importance that immunotherapeutic treatment start as early as possible (4, 8, 10). Second, immunotherapy triggered a weak immunological memory, insufficient to prevent the new infection but enough to enhance the response to the second treatment. An ELISA showed high levels of immunoglobulin G (IgG) antibodies following the second infection (130% above the cutoff point, versus 72% for the first infection). Anti-*Pythium* antibody is high in infected horses and usually increases during immunotherapy, which undoubtedly aids in successful treatment of the disease (10).

Oddly, the development of reinfection indicates that even in immunocompetent animals the antigenic patterns of hyphae and of the immunotherapy product are not enough to thwart new infections. Considering that oomycetes do not develop mutations, as do viruses, the mechanism of evasion of the immune response of the equine host is very efficient. *P. insidi-*



FIG. 2. (A) Equine with pythiosis in the shin region, May 2007. (B) The animal was considered cured following five doses of immunotherapy.



FIG. 3. (A) The same equine shown in Fig. 2 with abdominal presentation, December 2009. (B) The animal was considered cured following four doses of immunotherapy.

osum hyphae proliferate inside the kunkers, producing large quantities of exoantigens, therefore blocking the immune response (8, 9). Moreover, preventive immunotherapeutic characteristics have not been evidenced in field studies with treated and untreated animals exposed to the same environmental conditions where reinfection often occurs in previously treated animals (J. M. Santurio, unpublished data). As hypothesized by Gaastra et al. (4), such a finding could be explained by the fact that IgG antibody titers progressively decrease after the end of immunotherapy, which could protect the host for short periods of time (1 year). Notwithstanding, the use of different adjuvants elicits a stronger and longer immunologic response in rabbits experimentally infected by *P. insidiosum* (6). As the inoculation of *P. insidiosum* in species other than the rabbit has been consistently unsuccessful and observational studies are subjected to a limited number of researchers, the present study paved the way for the development of immunotherapeutic products with long-standing protective immunity.

We thank CAPES for conferment of the predoctoral scholarship and FAPEMAT for the project grant, process number 002.282/2007. We are grateful to D. G. Ubiali for histological examinations.

None of the authors of this paper has a financial or personal relationship with other people or organizations that could inappropriately influence or bias the content of the paper. Janio M. Santurio has a commercial interest in the immunotherapy product.

REFERENCES

1. **Botton, S. A., et al.** 2011. Identification of *Pythium insidiosum* by nested PCR in cutaneous lesions of Brazilian horses and rabbits. *Curr. Microbiol.* **62**: 1225–1229.
2. **Chaffin, M. K., J. Schumacher, and W. J. McMullan.** 1995. Cutaneous pythiosis in the horse. *Vet. Clin. North Am. Equine Pract.* **11**:91–103.
3. Reference deleted.
4. **Gaastra, W., et al.** 2010. *Pythium insidiosum*: an overview. *Vet. Microbiol.* **146**:1–16.
5. **Leal, A. B. M., A. T. Leal, J. M. Santurio, G. D. Kommers, and J. B. Catto.** 2001. Equine pythiosis in the Brazilian Pantanal region: clinical and pathological findings of typical and atypical cases. *Pesqui. Vet. Bras.* **21**:151–156.
6. **Leal, A. T., et al.** 2002. Serological response in rabbits immunized with *Pythium insidiosum* antigens associated with different adjuvants. *Cienc. Rural* **32**:1027–1032.
7. **Mendoza, L., F. Hernandez, and L. Ajello.** 1993. Life cycle of the human and animal oomycete pathogen *Pythium insidiosum*. *J. Clin. Microbiol.* **31**:2967–2973.
8. **Mendoza, L., and J. C. Newton.** 2005. Immunology and immunotherapy of the infections caused by *Pythium insidiosum*. *Med. Mycol.* **43**:477–486.
9. **Miller, R. I.** 1981. Treatment of equine phycomycosis by immunotherapy and surgery. *Aust. Vet. J.* **57**:377–382.
10. **Newton, J. C., and P. S. Ross.** 1993. Equine pythiosis: an overview of immunotherapy. *Compend. Contin. Educ. Pract. Vet.* **15**:491–493.
11. **Pereira, D. I. B., et al.** 2008. Comparison between immunotherapy and caspofungin as agents to treat experimental pythiosis in rabbits. *J. Mycol. Med.* **18**:129–133.
12. **Santurio, J. M., et al.** 2003. Three types of immunotherapies against pythiosis insidiosi developed and evaluated. *Vaccine* **21**:2535–2540.
13. **Santurio, J. M., S. H. Alves, D. B. Pereira, and J. S. Argenta.** 2006. Pythiosis: an emergent mycosis. *Acta Scientiae Veterinariae* **34**:1–14.
14. **Santurio, J. M., et al.** 2006. Indirect ELISA for the serodiagnostic of pythiosis. *Pesqui. Vet. Bras.* **26**:47–50. (In Portuguese.)